High soil fertility decreases sucrose content and susceptibility of loblolly pine roots to ectomycorrhizal infection by *Pisolithus tinctorius*

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Pinus taeda seedlings were grown for 10 weeks without ectomycorrhizae under low and high rates of complete soil fertility, as well as these rates minus N, P, K, or Ca. Seedling growth, inorganic chemical content of needles, and soluble-carbohydrate content of short roots were significantly affected by soil fertility, especially the high rates of N and P. Number and length of lateral and short roots were not affected by soil fertility. Sucrose and fructose contents of short roots were lowest at high levels of N and P; glucose was not detected. Seedlings from each of the 10 fertility combinations were inoculated with vegetative inoculum of *Pisolithus tinctorius* and incubated for 19 to 21 days at a moderate level of complete soil fertility. Significantly more ectomycorrhizae were formed on seedlings from the complete low fertility rate than on those from the high rates of N and P. Ectomycorrhizal development on seedlings from the other fertility combinations were intermediate from these extremes. Sucrose content of short roots determined before inoculation was significantly correlated with ectomycorrhizal development and accounted for 85% of the variation in susceptibility of short roots to infection by *P. tinctorius*. Fructose content of short roots was not correlated with ectomycorrhizal development. These results indicate that high levels of N and P in soil decrease sucrose content of short roots of loblolly pine and decrease their susceptibility to ectomycorrhizal development by *P. tinctorius*.

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Les auteurs ont cultivé des plantules de *Pinus taeda* pendant 10 semaines sans ectomycorrhize dans des sols de fertilité complète à des taux bas et élevés ainsi qu'avec ces amendements mais en supprimant, N, P, K, ou Ca. La croissance des plants, le contenu en éléments minéraux des aiguilles et le contenu en glucides solubles dans les racines courtes sont affectés par la fertilité du sol en particulier par les quantités élevées de N et P. Le nombre et la longueur des racines latérales et des racines courtes ne sont pas affectées par la fertilité du sol. Les contenus en sucrose et en fructose des racines courtes sont plus élevés avec les fortes quantités de N et P; le glucose ne peut être détecté. Des plantules provenant de chacune des 10 combinaisons de fertilité ont été ensuite inoculées avec du mycélium de *Pisolithus tinctorius* et incubées pendant 19 à 21 jours avec un sol de fertilité équilibrée et modérée. Dans ce cas, on retrouve alors plus d'ectomycorrhizes sur les plantules provenant d'une fertilisation complète mais faible que sur celles préalablement cultivées avec de fortes concentrations de N et P. La formation d'ectomycorrhizes sur les plantules provenant des autres combinaisons de fertilité se situe entre ces extrêmes. Le contenu en sucrose des racines courtes, déterminé avant l'inoculation, montre une corrélation significative avec le développement des ectomycorrhizes et explique 85% de la variation de susceptibilité à l'infection des racines courtes par le *P. tinctorius*. Le contenu en fructose des racines courtes ne montre pas de corrélation avec le développement des ectomycorrhizes. Ces résultats montrent que de fortes concentrations de N et P dans le sol diminuent le contenu en sucrose des racines courtes du *P. taeda* et diminuent ainsi leur susceptibilité au développement d'ectomycorrhizes sous l'influence du *P. tinctorius*.

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Introduction

Many factors influence ectomycorrhizal development on roots of trees (Harley 1969; Marks and Kozlowski 1973). The effect of soil fertility on internal concentrations of soluble carbohydrates in roots and their effect on the susceptibility of these roots to infection by ectomycorrhizal fungi, however, remains a controversial issue. Hatch (1937) proposed that the incidence of ectomycorrhizae on *Pinus* was controlled by the internal concentrations of nutrients, especially N, P, K, and Ca, in the roots. Later, Björkman (1942) found that N and P levels in soil influenced ectomycorrhizal development but concluded that the internal concentration of soluble reducing sugars in the roots accounted for differences in ectomycorrhizal development. Björkman proposed that a high level of soluble carbohydrates in root systems was a prerequisite to symbiotic infection; low levels decreased susceptibility. Harley and Waid (1955) analyzed roots of *Fagus* seedlings and reported results which supported Björkman's theory. Meyer (1962), Schweers and Meyer (1970), and Handley and Sanders (1962) concluded from their experiments with *Fagus*, *Picea*, and *Pinus* seedlings that the sugar content of roots resulted from the activity of the symbiotic fungi rather than the level of inorganic nutrient supply in the soil.

Harley (1969) criticized the methods for carbohydrate analyses used by Björkman and other workers because roots were analyzed for all reducing substances and these could contain a lot of impurities. However, he agreed with Hatch (1937), Björkman (1942), and many others that low nutrient levels in soil promoted ectomycorrhizal development. After the above criticism, Björkman (1970) reexamined his "carbohydrate theory" using Meyer's (1962) analytical techniques for assaying reducing substances. He grew *Pinus sylvestris* L. seedlings with ectomycorrhizae formed by two species of *Boletus* under different conditions of light and soil fertility and separately analyzed entire shoots and roots for reducing substances. He again concluded that high light or low fertility increased the quantity of reducing sugars in the roots and enhanced ectomycorrhizal development.

There are major problems in the cited reports. Most of the carbohydrate analyses included all types of tissue from large woody laterals and short roots. Since ectomycorrhizal fungi infect primary cortical tissues of short roots and some lateral root tips, the dilution of this primary tissue with large volumes of secondary root tissues in the carbohydrate analyses altered the reliability of the results. Furthermore, many of the previous workers assayed roots only for reducing substances, including carbohydrates. As pointed out by Harley (1969), these assays precluded the detection of other metabolically important sugars. Another problem is that soil fertility may have significantly influenced the survival and activity of the symbiotic fungi in the soil, reducing ectomycorrhizal development. Most of the cited workers did not report any statistical analyses of their data, and therefore, the validity of their conclusions is questionable. The most important deficiency was the failure to analyze roots before ectomycorrhizal fungus infection. Fungal infection undoubtedly changes carbohydrate makeup in short roots, and analysis after infection gives no indication of the sugars present before infection (Lewis and Harley 1965).

In the experiment described here, we assessed the role of soil fertility and root carbohydrates on ectomycorrhizal development in pine using techniques designed to avoid the mentioned problems. We (1) grew *Pinus taeda* L. seedlings in soil free of ectomycorrhizal fungi at different levels of N, P, K, and Ca; (2) determined seedling growth differences; (3) analyzed for soluble carbohydrates (reducing and nonreducing sugars) in nonmycorrhizal short roots and for nutrient contents of needles of representative seedlings; (4) inoculated other seedlings from each treatment with vegetative inoculum of *Pisolithus tinctorius* (Pers.) Coker and Couch, and incubated these seedlings in a moderately fertile soil; and (5) made complete statistical analyses of all data.

Materials and Methods

This study was conducted in an electronically air-filtered and air-conditioned plant growth room in Athens, Georgia. All materials and equipment used were either fumigated with depolymerized paraformaldehyde in the growth room or sterilized before use in the room (Marx 1973). All seedlings grown received about 75% of full sunlight for a 14- to 16-h photoperiod. Air temperature varied between 19 and 31°C, and soil temperatures varied between 19 and 26°C.

Seedling Predisposition Phase

Forty styrofoam units (Styroblock 8®), each with 20 100-cm³ seedling cavities, were filled with a mixture of

TABLE 1. Low and high fertility levels used to predispose loblolly pine seedlings to infection by *Pisolithus tinctorius*

Treatment	Nitrogen, ppm	Phosphorus, ppm	Potassium, ppm	Calcium, ppm
Complete low fertility (CLF)	10	10	10	10
Low fertility – N (LF–0N)	0	10	10	10
Low fertility – P (LF–0P)	10	0	10	10
Low fertility – K (LF–0K)	10	10	0	10
Low fertility – Ca (LF–0Ca)	10	10	10	0
Complete high fertility (CHF)	300	75	100	80
High fertility – N (HF–0N)	0	75	100	80
High fertility – P (HF–0P)	300	0	100	80
High fertility – K (HF–0K)	300	75	0	80
High fertility – Ca (HF–0Ca)	300	75	100	0

equal volumes of forest clay soil and sand which had been triple steamed. Available nutrients in the mixture were 10 ppm P, 30 ppm K, 136 ppm Ca, 20 ppm Mg, 5 ppm NO_3-N, with a reaction of pH 6.0.[2] Several loblolly pine seeds from a mixed seed lot were planted in each seedling cavity. Before planting, seeds were soaked for 48 h at 5°C in 1% H_2O_2 and then were surface-sterilized for 15 min in 30% H_2O_2. The seedlings were thinned to one per cavity 3 weeks after planting. One week after thinning, the 40 seedling units were randomly arranged into 10 groups of 4 seedling units each. Each group of four containers (80 seedlings) was randomly assigned to 1 of 10 fertility treatments (Table 1).

The chemical sources of N, P, K, and Ca were reagent grade NH_4NO_3, Na_3HPO_4, KCl and $CaCl_2$. A micronutrient solution containing Mg, Mo, Cu, B, Zn, Mn, and chelated Fe was added to all nutrient solutions (Rowan and Steinbeck 1977). The nutrient solutions were prepared just before use and each was adjusted to pH 5.5 with either 1% HCl or NaOH. Each of the 800 test seedlings received 25 ml of assigned nutrient solution 2, 5, and 8 weeks after thinning. Seedlings were watered with tap water as needed. The experimental design was a randomized complete block with 10 fertility treatments. Each treatment had four replicate seedling container units; each unit contained 20 pine seedlings.

Two weeks after the last application of nutrients, 10 randomly selected seedlings were removed from each container unit, and the soil mixture was gently washed from the roots. Height, number and length of lateral roots, number of short roots, and shoot and root dry weights (dried for 96 h at 45°C) were obtained from five of these seedlings. Roots were measured and counted under 4 × magnification. One-hundred milligrams (fresh weight) of short roots were excised from the remaining five seedlings per treatment replicate and placed immediately into glass vials containing 50% ethanol at 40°C for carbohydrate analyses. The needles of these seedlings were dried and combined with needles of the former five seedlings for inorganic chemical analyses.

[2]All inorganic chemical analyses reported herein were done by the Soil Testing and Plant Analysis Laboratory, Extension Service, University of Georgia, Athens, GA, U.S.A. 30602.

Carbohydrate Analysis of Short Roots

Short roots (100 mg) in 50% ethanol were homogenized and centrifuged (27 000 × g). The supernatant was decanted to separate cell debris and polysaccharides from the soluble carbohydrates. Ethanol was removed by lyophilization and samples were dissolved in 10 ml of water. Samples were deionized by passage through Dowex 1 – HCO_3^- column (2.2 × 4 cm), followed by passage through Dowex 50 – H^+ column (2.2 × 4 cm). The pH was maintained at 7.5 with 0.01 N sodium phosphate buffer. Sucrose content was determined by a modification of the method of Roe (Ashwell 1957). Fructose content was determined by difference after treating the extract with 0.2 N NaOH for 10 min at 100°C to destroy reducing sugars (Mendicino 1960). Total carbohydrate content was determined by the phenol – sulfuric acid method (Montgomery 1961) with fructose and glucose standards. Glucose content was determined with glucose oxidase (Avigad *et al.* 1962) and the less specific Park–Johnson method (Park and Johnson 1949).

Ectomycorrhizal Development Phase

The remaining 10 seedlings in each of the four container units per treatment were removed, and the soil mixture was washed from the roots. Roots of five seedlings were dipped individually into a water slurry of leached vermiculite – peat moss containing vegetative inoculum of *Pisolithus tinctorius* (Marx and Bryan 1975). The slurry was a 1 : 1 by volume ratio of water and inoculum. Care was taken to assure that the entire root systems were coated with vermiculite particles containing *Pisolithus* mycelia. Seedlings were then transplanted into a steamed soil mixture in individual 1-ℓ polyethylene plastic pots. The remaining five seedlings per container unit were treated as above except that the water slurry contained autoclaved inoculum of *Pisolithus*. The potted seedlings were randomly placed on a bench in the growth room and each was fertilized with 100 ml of a solution containing 30 ppm N, 20 ppm P, 25 ppm K, 30 ppm Ca, and micronutrients. Seedlings were watered with tap water as needed.

After 19 to 21 days incubation, seedlings were removed and growth measurements taken as previously described. This incubation period was long enough to obtain a sufficient number of ectomycorrhizae for an accurate assessment but short enough to minimize changes in the

TABLE 2. Growth measurements, inorganic chemical analyses of needles, and root carbohydrate analyses of nonmycorrhizal loblolly pine seedlings after 10 weeks at 10 levels of soil fertility and their susceptibility to ectomycorrhizal infection by *Pisolithus tinctorius* (*Pt*) after predisposition by soil fertility*

Parameter	CLF	LF−0N	LF−0P	LF−0K	LF−0Ca	CHF	HF−0N	HF−0P	HF−0K	HF−0Ca
					Growth measurements					
Height (cm)	7.6a	8.2a	8.0a	7.8a	7.5a	11.2b	7.7a	9.3ab	10.6b	11.6b
Top dry weight (mg)	114a	137a	148a	150a	138a	432c	124a	210b	282bc	402c
Total dry weight (mg)	151a	174a	193a	195a	182a	519c	168a	266b	343b	495c
					Needle analyses (mg/gm tissue)					
Nitrogen	10.3a	8.7a	9.2a	8.9a	10.0a	17.3b	8.5a	15.8b	20.1c	20.7c
Phosphorus	2.7a	2.5a	1.9b	2.4a	2.3a	3.2c	3.3c	1.5b	3.3c	3.7c
Potassium	9.7a	10.5a	10.0a	10.1a	11.0a	11.2a	13.1b	9.8a	9.9a	11.8ab
Calcium	2.2a	2.5a	2.6a	2.3a	2.5a	1.8b	3.5c	1.3b	1.7b	1.4b
					Root carbohydrate analyses (μmol/g short root)					
Sucrose	0.75a	0.66b	0.62b	0.63b	0.52bc	0.38d	0.63b	0.50c	0.47c	0.45c
Fructose	1.56a	1.50a	1.84b	1.69ab	1.50a	1.49a	1.74b	1.84b	1.98b	1.88b
					Ectomycorrhizal infection					
Percentage *Pt* ectomycorrhizae	19.0a	13.2b	14.1b	13.1b	11.4b	3.5c	9.6b	9.2b	4.0c	5.8bc

*Each number followed by a common letter (a, b, or c) within a parameter is not significantly different at the $P = 0.05$ confidence level.

biochemical status of roots induced before infection. To confirm formation of ectomycorrhizae, suspected short roots were removed from lateral roots, cut repeatedly with a razor blade on a glass slide, mounted in lacto-phonol–phloxine dye (1 mg dye in 50 ml lactophenol) under a cover slip, and examined microscopically at 100 × magnification. Presence of the Hartig net or fungus mantle was considered evidence of ectomycorrhizal development. Morphology of short roots could not be used as a diagnostic feature for ectomycorrhizae as over half of the short roots from both the control and inoculated seedlings were bifurcate to coralloid. An infected short root was counted as one ectomycorrhiza regardless of the amount of dichotomy. Percentage ectomycorrhizae on each seedling was calculated by dividing the number of feeder roots (short roots + lateral roots + ectomycorrhizae) into the number of ectomycorrhizae and multiplying the quotient by 100.

Analyses of variance were made on all seedling growth and carbohydrate data, and significant differences were evaluated with Duncan's multiple-range test at the 95% confidence level.

Results

Seedling Growth Data

Seedling growth after 19 to 21 days with *Pisolithus* was not different from growth that occurred before inoculation with *Pisolithus* in any fertility regime. Control seedlings inoculated with killed *Pisolithus* inoculum were not significantly different from inoculated seedlings. These control seedlings were free of ectomycorrhizae from accidental contamination, an indication that the ectomycorrhizae on seedlings treated with active *Pisolithus* resulted from inoculation.

Soil fertility did not influence the number ($\bar{X} = 28 \pm 9$) of short roots or total length of lateral roots ($\bar{X} = 378 \pm 99$) of seedlings. Therefore, all seedlings had about the same number of short roots when inoculated with *Pisolithus*.

None of the low fertility regimes affected height, dry weight of shoots, or total dry weight of seedlings (Table 2). Obviously there were sufficient quantities of these nutrient elements in the original soil mixture to offset possible deficiencies influencing seedling growth. High fertility combinations influenced seedling growth. In the HF − 0N regime,[3] seedlings grew poorly and were similar in height and weight to those grown in low fertility. HF − 0P and HF − 0K treatments also reduced seedling growth significantly. Seedlings from the HF − 0Ca treatment were as large as those from the CHF treatment.

[3]For explanation of regimes, see Table 1.

Soil fertility treatments had significant effects on the quantity of test elements in needles of seedlings (Table 2). Less P was detected in needles of seedlings from the LF − 0P regime. Other fertility combinations in the low fertility tests did not change N, P, K, or Ca status in needles. Less N and P were detected in needles of seedlings from the HF − 0N and HF − 0P treatments. Seedlings from the HF − 0N treatment had more K and Ca in needles than did other seedlings. Presence or absence of added K or Ca did not change the K or Ca status of needles at either fertility level. Quantities of Mg, Mn, Fe, B, Al, Sr, Ba, and Na in needles were not affected by the soil fertility treatments. These results on foliar analyses agree with those of Fowells and Krauss (1959).

Carbohydrate Analyses of Short Roots

Quantities of sucrose and fructose in short roots were affected by soil fertility (Table 2). Frustose levels in short roots exceeded those of sucrose in seedlings grown in the LF − 0P treatment and in all the high fertility treatments, except for CHF. Fructose contents of short roots were similar in the CLF and CHF treatments. There was more sucrose in short roots from seedlings grown in the CLF treatment than in other treatments. Slightly less sucrose was detected in short roots from seedlings grown in the LF − 0N, LF − 0P, LF − 0K, LF − 0Ca, and HF − 0N treatments. Considerably less sucrose was detected in the HF − 0P, HF − 0K, and HF − 0Ca regimes. Short roots from seedlings grown in the CHF treatment contained significantly less sucrose than short roots from any other fertility combination.

Glucose was not detected in any short roots. The adequacy of the technique for detecting glucose was demonstrated by adding reagent grade glucose to extra samples of short roots. Over 90% of the added glucose was detected by the glucose oxidase technique.

Ectomycorrhizal Development

Soil fertility affected susceptibility of short roots to ectomycorrhizal development by *P. tinctorius* (Table 2). Over four times more ectomycorrhizae were formed on seedlings previously grown in the CLF treatment than on seedlings grown in the CHF, HF − 0K, and HF − 0Ca (high quantities of N and P). Seedlings from the other fertility treatments in both low and high

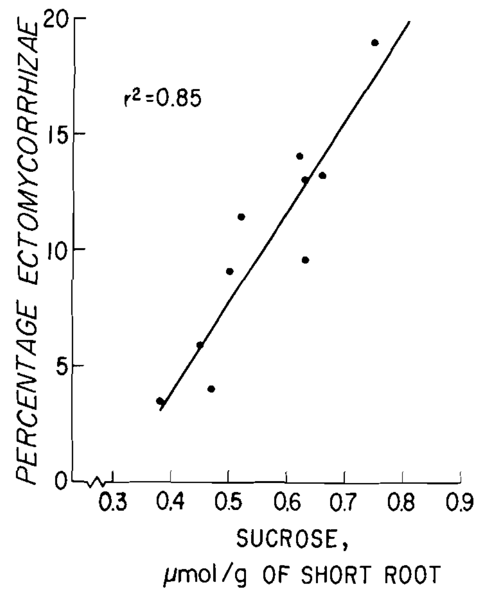

FIG. 1. Correlation between sucrose content of short roots of 10-week-old loblolly pine seedlings maintained under 10 levels of soil fertility and percentage ectomycorrhizae formed after inoculation with *Pisolithus tinctorius*.

regimes with certain elements deleted were intermediate in ectomycorrhizal development and were significantly different from the above extremes.

A correlation between quantities of sucrose in short roots and the percentage of short roots infected by *Pisolithus* was apparent (Fig. 1). Analysis showed that sucrose content of short roots accounted for 85% of the variation in ectomycorrhizal development. Fructose content of short roots was not correlated with percentage ectomycorrhizae (r^2 = 0.36).

Discussion

We found a negative correlation between high levels of N and P and susceptibility of short roots of loblolly pine to ectomycorrhizal infection by *Pisolithus tinctorius*. These results confirm the earlier reports of Hatch (1937), Björkman (1942, 1970), and others (Harley 1969). However, we also found a negative correlation between high levels of N and P and the sucrose content of short roots. Sucrose content accounted for 85% of the variation in *Pisolithus* infection of these short roots. In contrast with the reports of Björkman (1942, 1970), we did not find a significant correlation between the quantity of

fructose (a reducing sugar) in short roots and the susceptibility of these roots to ectomycorrhizal infection. However, our findings do support Björkman's concept that the susceptibility of short roots to ectomycorrhizal infection is increased as the concentration of soluble sugars increases.

We concluded that the sugar in the soluble-carbohydrate pool in short roots of loblolly pine that influences susceptibility to ectomycorrhizal infection by *Pisolithus* is not a reducing sugar, as Björkman proposed, but is the nonreducing sugar, sucrose. Our data support the suggestion of Lewis (1975) that the major carbon source in roots which predisposes them to infection by ecologically obligate biotrophs is the main translocated carbohydrate in higher plants . . . sucrose.

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